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THE UNIVERSITY OF ALBERTA

MOLECULAR CLONING OF THE HUMAN LIPOXYGENASE GENE

by

(C)

SARA GHARAVI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MOLECULAR CLONING OF THE HUMAN LIPOXYGENASE GENE submitted by SARA GHARAVI in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

.....

(Supervisor)

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.....

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Date: June 30, 1986.

To my family whose support

across the miles has been a

constant encouragement.

ABSTRACT

Three human genomic clones, homologous to a rabbit reticulocyte lipoxygenase cDNA, were isolated from a human: λEMBL3 library and characterized. Initial analysis of the structural organization revealed a large region of overlap between the inserts. It is proposed that cloned lipoxygenase sequences represent the same region of the genome. Mapping of the clones was carried out using single and double digestion with restriction endonucleases as well as a novel Southern Cross mapping system. An 8.5 kb region from two of the isolated clones and a 5.5 kb region from the third, were assigned as part of the transcriptional unit of the lipoxygenase gene. The precise delineation of the exon/intron regions cannot be defined as a full length cDNA is not available. Southern transfer of human genomic DNA was performed to identify any rearrangement of the sequences during the cloning procedure; none was observed. The identity of the human inserts was verified by hybridization of specific human DNA probes to lipoxygenase mRNA from rabbit and human reticulocyte poly(A)$^+$ RNA fractions. The probe does not select any message from a T-lymphoma cell-line, indicating the likely specificity of the lipoxygenase expression in reticulocytes.

In view of the appearance of certain weakly hybridizing bands in genomic Southern transfer, and the diversity of the enzyme activity in blood cells in general, the possible existence of a multigene family is suggested for lipoxygenase.

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INTRODUCTION

Maturation of the red blood cells

All types of blood cells are derived from a common progenitor called the haematopoietic stem cell. Under the influence of various growth factors (including the hormone erythropoietin) and the microenvironment, the erythroid precursors of red blood cells (RBC) undergo characteristic morphological changes and start to synthesize RBC proteins. These include structural proteins which constitute the cytoskeleton of these cells and various other proteins responsible for the functioning of the RBC (for review see Harrison, 1984).

A selective fraction of the stem cells in the bone marrow become "committed" and are converted to erythrocytes. Differentiation from stem cells to mature erythrocytes takes about 6 days; the reticulocyte stage occupies half this period. The absence of a nucleus in the RBC of mammals and nuclear inactivity in those of lower vertebrates marks this stage while active respiration and protein synthesis are concurrent with the degeneration of endoplasmic reticulum (only vestigal remnants of the network remain). Lysosomes almost completely disappear. The transition to the erythrocyte stage is characterized by drastic changes in the reticulocytes. Initially the mitochondria disintegrate; this event is followed by the loss of respiratory activity as well as ribosomes and most of the receptors and transport proteins of the cell membrane (Rapoport *et al.*, 1979). At the same time, the synthesis of globin increases while most other proteins disappear.

In reticulocytes, inhibition of respiration (by action on the

iron-sulphur regions of the respiratory chain) was attributed initially to a protein known as Factor F. This activity was detected in stroma-free haemolysates of reticulocytes from rabbits and in much smaller amounts in blood cells from anaemic chickens. None of this inhibition was observed in red cells from non-anaemic blood. A similar inhibitor, characterized by its action on the ionized form of cytochrome oxidase, was associated with a Factor C (Rapoport *et al.*, 1974). Finally, a third factor responsible for the mitochondrial membrane lysis was identified (Schewe *et al.*, 1975). However, Rapoport *et al.* (1979) demonstrated that all three activities were found in a single protein called lipoxygenase. This enzyme is one of the most abundant non-haemoglobin proteins synthesized in reticulocytes. It constitutes about 3-4% of the newly-synthesized cytosolic proteins including globins and approximately 30% of the non-haemoglobin proteins (Thiele *et al.*, 1979).

Reticulocyte lipoxygenase enzyme system

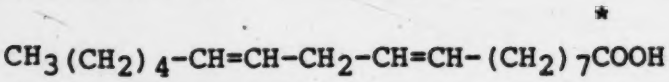
Rabbit lipoxygenase (EC.1.13.12) from reticulocytes is a single polypeptide glycoprotein (5% neutral sugars) with a molecular weight of 78,000 daltons, containing one non-heme iron atom per enzyme molecule. The amino acid content analysis depicts a high percentage of leucine and tryptophan in the protein with a glycine molecule at the N-terminus and at least 3 but not more than 5 peptide chains with identical N-termini, differing in C-terminal amino acids (Rapoport *et al.*, 1979). This cytosolic lipoxygenase initiates the disintegration of the mitochondria by oxygen insertion into the fatty acid chains of their membranes. The attack triggers an ATP-dependent proteolysis of

mitochondria which precedes the final degradation of the organelle by proteases and other phospholipids. It has been shown that the susceptibility of the mitochondria to the lipoxygenase-ATP-dependent proteolytic system is conferred by an Fe-dependent protein called mitochondria susceptibility factor (Rapoport *et al.*, 1986) as well as the maturational state of the reticulocytes (Rapoport *et al.*, 1985). Erythrocyte ghosts are much more resistant than mitochondrial membranes possibly due to their high content of cholesterol and to specific protein lipid interactions. Apparently the barrier lies in the interior of the red cell membrane since spectrin depletion does not cause the susceptibility of the membrane towards lipoxygenase (Rapoport *et al.*, 1979). A marked property of lipoxygenase is the suicidal character of its action on lipids and sub-mitochondrial particles. The inactivation is irreversible since the addition of substrate (linoleic acid, Fig. 1A) does not restore the activity.

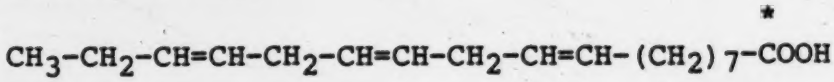
Extensive studies of the arachidonic acid metabolism by purified rabbit reticulocyte lipoxygenase has been carried out in intact rabbit reticulocytes. This unsaturated fatty acid (Fig. 1C) is the major substrate for other lipoxygenases of the reticuloendothelial cells. The products of the enzyme activity include 15-HPETE (15-hydroperoxyeicosatetraeonic acid) and 15-HETE (15-hydroxyeicosatetraeonic acid) indicating that the enzyme is specific for C-15 (n-9) oxygenation. Moreover, judging by the high amounts of 15-HETE, it is apparent that the reticulocyte lipoxygenase is coupled to a peroxide system (Bryant *et al.*, 1982). In addition, a 12-lipoxygenase activity has been detected which co-purifies with the 15-lipoxygenase enzyme (Kuhn *et al.*, 1983).

Figure 1 Examples of unsaturated fatty acids that serve as substrates for lipoxygenase action (A-C). (C) also indicates the metabolites of 15-lipoxygenase activity. C-1 is indicated by C* (White *et al.*, 1964).

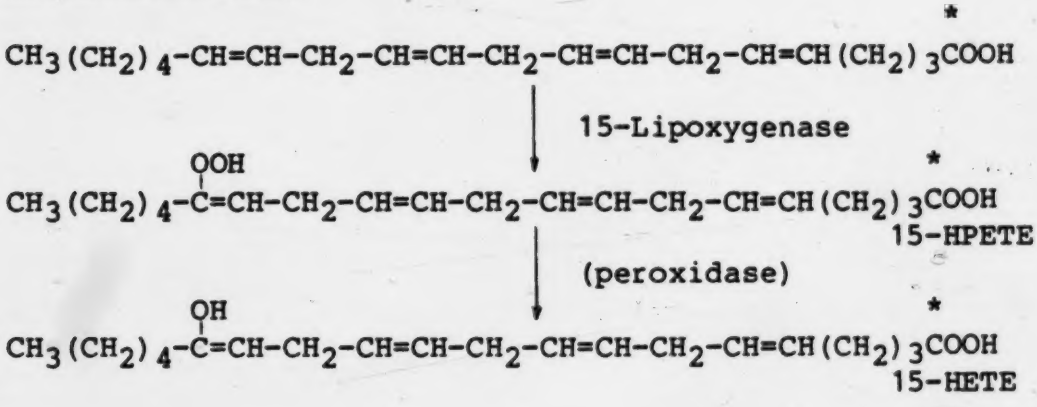
Linoleic Acid (A)



Linolenic Acid (B)



Arachidonic Acid (C)



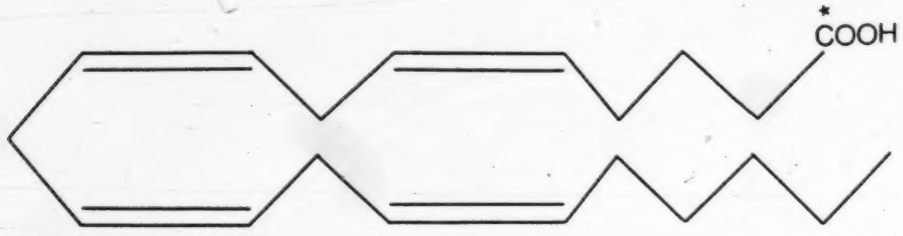
Lipoxygenase in non-RBC cells

Lipoxygenases are widely distributed in plants and animals and, depending on the source, vary in their substrate specificity, pH optima, susceptibility to inhibitors and the isomeric nature of their products.

In plants, the purified lipoxygenase from soya bean can be resolved into at least 4 subspecies that vary in their optimum pH and product specificities (Parker, 1984). On the other hand, the single enzyme from potato tubers has been shown to possess both 5- and 8-lipoxygenase activities (Shimizu *et al.*, 1984). Recently, the cloning of a lipoxygenase cDNA from pea seedlings has been reported (Casey *et al.*, 1985).

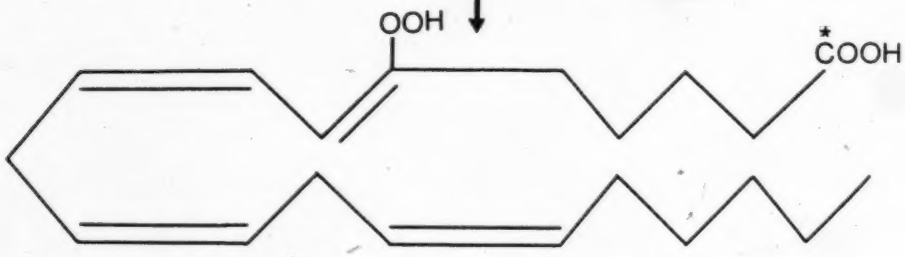
Three major lipoxygenase pathways have been identified in mammalian tissues. The 5- and 15-lipoxygenases are of predominantly granulocyte origin (Borgeat *et al.*, 1976); the 12-lipoxygenase is platelet-derived (Hamberg and Samuelsson, 1974). There is also a minor 15-lipoxygenase pathway in platelets as shown by the metabolites of the enzyme action on arachidonic acid (Wong *et al.*, 1985). On the other hand, there is disagreement about whether lymphocytes produce 5-lipoxygenase metabolites (Parker *et al.*, 1979; Golydne *et al.*, 1984). The 5-lipoxygenase from leukocytes seems to be unique amongst other blood cells. This enzyme requires stimulatory factors for its activity which comprise at least 3 components; two of them soluble and one membrane-bound, in addition to Ca^{++} and ATP. The complex regulation of this enzyme may play a role in the synthesis of leukotrienes (Fig. 2) and lipoxins (Rouzer and Samuelsson, 1985; Rouzer *et al.*, 1985). These recently characterized compounds are of biological importance

Figure 2 Leukotriene formation in human leukocytes by 5-lipoxygenase pathway of arachidonic acid metabolism. * denotes C-1 (Samuelsson, 1983).



Arachidonic Acid

5- Lipoxygenase



5- hydroperoxy 6,8,11,14 eicosatetraenoic acid **(5-HPETE)**

Dehydrase

Leukotriene A4

Hydrolase

Glutathione-S-transferase

Leukotriene B4

Leukotriene C4

Glutamyl transpeptidase

Leukotriene D4

Leukotriene E4

γ-Glutamyl transpeptidase

Leukotriene F4

since they are involved in inflammation and immediate hypersensitivity (Samuelsson, 1983). Mediation of leukotrienes in reversible airway disease in bronchial asthma as well as airway hyperirritability to other agonists and irritants has been established (Austen and Lewis, 1984).

Post-transcriptional regulation

Rabbit reticulocyte lipoxygenase has not been detected in cells restricted to bone marrow; its synthesis seems to be induced when reticulocytes reach the peripheral blood, since no antigenically-related protein such as a precursor molecule is found in nucleated blood cells. It is therefore postulated that the transcription of the lipoxygenase mRNA takes place in the nucleated bone marrow cells but the transcripts are translationally inactive (Thiele *et al.*, 1982). It has been shown that this mRNA exists as cytoplasmically free ribonucleoprotein particles (mRNP). Primary transcripts in the nucleus and fully processed mRNAs in the cytoplasm are found as RNPs and several roles, including nucleoplasmic transport of mRNA, *in vivo* stability of mRNA, and regulation of translation have been assigned to these particles. Two main mechanisms of translation inhibition have been suggested: By the protein complex in the free mRNP or by a certain species of low molecular weight "translational control" RNA (tcRNA) in the particles (Sarkar, 1984). Messenger RNA control of this nature has been studied in several tissues; rabbit reticulocytes (Blobel, 1972), duck erythroblasts (Vincent *et al.*, 1980) and *Artemia* (brine shrimp) embryos (Wahba and Woodley, 1984) are a few examples.

The rationale

The main objective of this investigation was the isolation and characterization of the human reticulocyte lipoxygenase gene from a genomic library by virtue of homology to a rabbit reticulocyte lipoxygenase cDNA. The interest regarding this work is two-fold:

a. As discussed earlier, the lipoxygenase pathway is an important alternative for arachidonic acid metabolism. The non-prostaglandins products of this pathway, the leukotrienes, are associated with inflammation in tissues. No doubt a better understanding of lipoxygenase structure (at the DNA level) and the relationship to its function could facilitate the study of leukotriene production and control from a pharmaceutical point of view.

Even though dual positional activity and substrate flexibility has been demonstrated for a few lipoxygenases, it is believed that the susceptibility of a fatty acid to the enzyme attack depends on the specific location of its double bonds and the lipoxygenase. The possibility of isozymes in certain cases has not been ruled out. However, efforts to distinguish heterogeneity at the DNA level from other bases for heterogeneity (post-translational modifications) have seldom been attempted.

b. Precisely due to this lack of information pertaining to the genomic organization of lipoxygenase, the enzyme system presents a challenge to geneticists and biochemists alike.

Studies on soya bean lipoxygenase indicate an independent assortment of at least 2 types of the enzyme, lipoxygenase-1 and

lipoxygenase-2 (Casey *et al.*, 1985) indicating there are a minimum of 2 loci for lipoxygenase in soya bean.

The relationship of lipoxygenases from different blood cells has not been well characterized. As first steps in resolving this problem, the protein has been purified from rabbit reticulocytes (Schewe *et al.*, 1975; Rapoport *et al.*, 1979) and human leukocytes (Rouzer *et al.*, 1985). Further studies regarding the mode of action of this enzyme are underway.

Do a group of related but non-identical genes code for different lipoxygenases? "Variant" repetition of genes has been shown for globin, actin and several other proteins (Long and David, 1980). In view of the data obtained during the course of this work, the possible existence of a gene family for lipoxygenases has been addressed.

Moreover, the mode of post-transcriptional regulation for lipoxygenase has not been determined. The red blood cell is well synchronized in switching on the synthesis of globin long before the destructive activity of the lipoxygenase has been detected. What roles do the protein components and tcRNA play in the inhibition of the translation? What factors are responsible for the induction of lipoxygenase synthesis?

Although the research relating to these questions is beyond the scope of this work, there is potential for further investigation in the future.

MATERIALS AND METHODS

I. CONSTRUCTION OF THE HUMAN GENOMIC LIBRARY

1. Preparation of host bacteria

A single bacterial colony was inoculated in 50 ml of LB medium containing 1% NaCl, 0.5% Bacto Yeast extract and 1% Bacto Tryptone. The medium was adjusted to pH 7.5 with 1N NaOH and supplemented with 0.2% (w/v) maltose. Following 12-16 hours of incubation at 37°C in a rotary shaker, the cells were centrifuged at 2800xg for 10 minutes at room temperature. The cell pellet was resuspended in sterile 0.01M $MgSO_4$ (0.4x volume of the original culture) and stored at 4°C.

2. Purification of bacteriophage λ

Plate lysate method (Huynh *et al.*, 1984): 125-250 µl of host bacteria (prepared as described above) was incubated with $2.5x10^6$ plaque forming units (pfu) of the bacteriophage lysate for 20 minutes at 37°C to facilitate phage adsorption to bacterial cells. The bacteriphage stocks were diluted in sterile SM solution consisting of: 100mM NaCl, 10mM $MgSO_4$, 50mM Tris.HCl (pH 7.5) and 5 ml of 2% (w/v) gelatin. Three ml of TM medium (1% Bacto Tryptone, 0.5% NaCl, 10mM $MgSO_4$ and 1% glucose) containing 0.7% Bacto Agar or agarose (at 46°C) were added to the mixture, vortexed and plated on TM plates (with 1.2% Bacto Agar). After 6-10 hours of growth at 37°C and confluent lysis, the plates were flooded with 5-10 ml of SM solution and cooled overnight at 4°C. The overlay solution was collected and the bacterial

debris removed by centrifugation at 9000xg for 15 minutes at 4°C. The phage were pelleted from the supernatant using an SW 27 rotor (Beckman) at 70,000xg for 90 minutes at 4°C. The bacteriophage particles were resuspended in 1 ml of SM and then purified using CsCl density gradient centrifugation. The phage suspension was brought to 0.75 gm CsCl/ml of the supernatant and spun at 180,000xg for at least 6 hours in a VTi 65 rotor (Beckman) at 4°C.

3. Extraction of bacteriophage λ DNA

The procedure followed is essentially that of Maniatis *et al.* (1982). After the removal of the CsCl by dialysis against TE buffer (10mM Tris.HCl pH 8 and 1mM EDTA), the phage suspension was treated with 20mM EDTA, 50 µg/ml proteinase K and 0.5% (w/v) SDS and incubated for 1 hour at 65°C. An equal volume of TE-saturated phenol was added,and following thorough mixing, centrifuged at 1600xg for 5 minutes at room temperature. The aqueous phase was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged, and the recovered aqueous phase extracted with 1 volume of chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume 3M Na acetate (pH 6) and 2-1/2 volume 95% (v/v) ethanol and stored at -20°C. Alternatively, the final aqueous phase was dialyzed against TE buffer overnight and then ethanol precipitated as above.

4. Human DNA extraction

Human DNA was extracted using the procedure of Maniatis *et al.* (1982) with modifications. 1-2 gm of frozen (-70°C) spleen tissue was ground to a fine powder in liquid nitrogen and then homogenized in

TABLE 1 Bacteria and bacteriophage strains

Strain	Markers	Source
E. coli		
NM 538	*sup* F, *hsd* R	Promega Biotec
NM 539	*sup* F, *hsd* R, P2 *cox* 3	Promega Biotec
Bacteriophage		
λEMBL3	*trp* E derivative of λ1059 [hλs BamI° b189 (*int* 29, NL 44, cI857, *pac* I29) (*int* -cIII) KH54, SRI4°, NIN5, *chi* 3]	Promega Biotec
λcI857	cI857	W. C. Clark

20 ml of 100mM EDTA (pH 8) and 150mM NaCl using a Wheaton glass homogenizer. Proteinase K and SDS were added to a final concentration of 100 μg/ml and 2%, respectively. The homogenate was incubated at 45°C for 1 hour after which the DNA was extracted with phenol as above.

5. Endonuclease digestion of DNA

All DNA samples were restricted with endonucleases using the manufacturer's specified conditions. The reaction mixtures were incubated at 37°C for 1-1/2 to 4 hours and the reactions stopped by the addition of 10mM EDTA (pH 8).

6. Gel electrophoresis

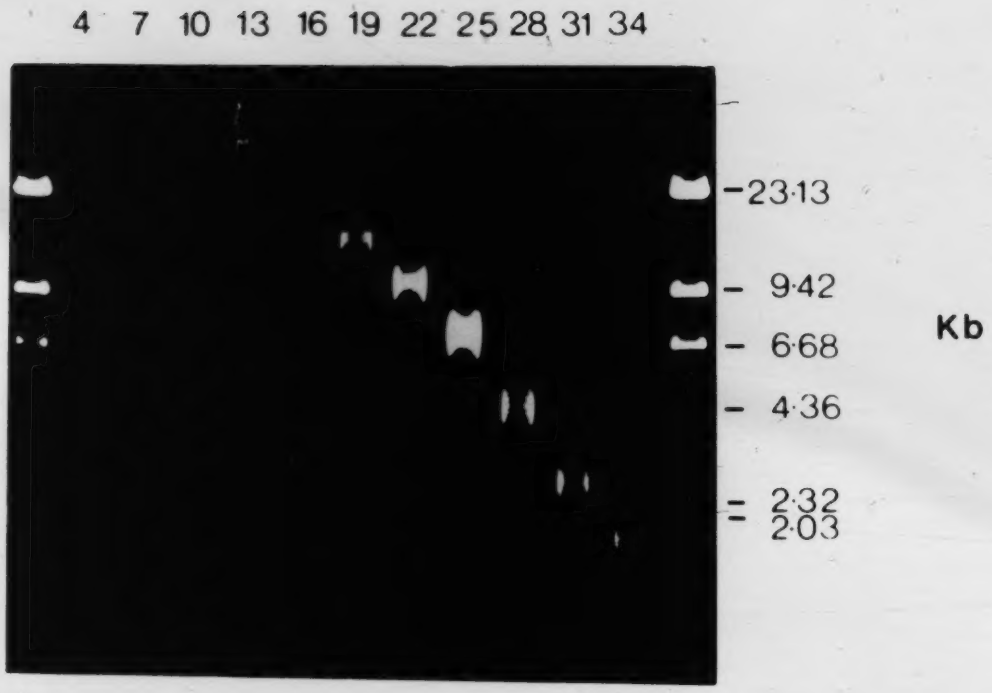
DNA samples were electrophoresed in 0.4% agarose (Ultra Pure, BRL) gels containing 40mM Tris acetate, 2mM EDTA (pH 8) in horizontal slab gel electrophoretic units (Tyler Research). Loading dye [0.15% (w/v) BPB, 0.15% (w/v) xylene cyanol and 50% (v/v) glycerol] was added to each sample. By the addition of ethidium bromide (0.5 μg/ml) to the agarose gel just prior to pouring, the DNA samples were visualized on a long wave UV transilluminator.

7. Sucrose gradient fractionation

Human DNA was digested with limiting amounts of *Sau* 3A and size-fractionated on a 10-40% linear sucrose gradient following the method described by Maniatis *et al.* (1982). Human DNA samples were heated in a 68°C incubator for 10 minutes, gently layered on the sucrose gradients and centrifuged at 70,000xg for 24 hours at 15°C. Gradients were fractionated by puncturing the bottom and dripping 0.5

Figure 3 Gel electrophoresis of human fractionated DNA. Approximately 250 μg of human spleen DNA was subjected to partial digestion with 0.15 unit of Sau 3A restriction endonuclease for 60 minutes and subsequently loaded onto a linear sucrose gradient. Following centrifugation, a sample of every third fraction was run in a 4% agarose gel and visualized by long wave UV transillumination. Molecular size markers were generated by *Hind* III digestion of bacteriophage λ. Fractions 10-22 were pooled for the purpose of library construction.

FRACTION #



ml fractions. A sample of every third fraction was electrophoresed using a 0.4% agarose gel (Fig. 3). Fractions containing 9-22 kilobase (kb) sized fragments were pooled and dialyzed for several hours against TE buffer and passed through a Nacs Prepac Column (Bethesda Research Laboratories) as described by the manufacturers. The columns were washed with 3 ml of 2M NaCl in TE (elution buffer) and equilibrated with 0.5M NaCl in TE (load buffer). The sample was applied to the column with 1 ml of load buffer and washed with 3 ml of the same buffer. The DNA was finally eluted with 1.5 ml of elution buffer and ethanol/Na acetate precipitated overnight.

8. Ligations

Ligation reactions were set up as proposed by Maniatis *et al.* (1982). After growth on permissive host NM 538 *E.coli* strain (Table 1), the λEMBL3 DNA was purified, double-digested with *Bam* HI and *Eco* RI and used as the vector. Prior to ligation with human DNA inserts, the cohesive sites of the phage arms were annealed in 10mM $MgCl_2$ for 1 hour at 42°C. A series of test ligations and *in vitro* packaging reactions was performed to determine the ratio of arms to inserts that yielded the highest number of packagable molecules. Each reaction contained various ratios of phage DNA to human DNA inserts [1(arms):1(insert), 2:1, 4:1 and 6:1] in ligation buffer consisting of 60mM Tris pH 7.5, 5mM $MgCl_2$, 5mM DTT and 1mM ATP. 1-2 units of T_4 DNA ligase was added and the reaction incubated for 12-16 hours at 12°C. As a negative control, a ligation reaction was set up without human DNA.

9. *In vitro* packaging

The resultant recombinant DNA molecules were packaged *in vitro* using single packaging extracts (Enquist and Lands, 1979; Hohn, 1979) obtained from Promega Biotec (PackaGene System). 10 μl of the ligation mixture was added to 50 μl of the extract after which the reaction was allowed to proceed for three hours at room temperature. The recombinant phage particles were then diluted in SM buffer and plated on *E.coli* strain NM 539 restriction host as described previously using 22x22 cm Nunc culture dishes (Gibco).

10. Amplification

For long term storage, the λEMBL3:human library was amplified (as described by Maniatis *et al.*, 1982). After 10 hours of incubation at 37°C, the plates were overlayed with 20 ml of sterile SM buffer and incubated overnight at 4°C. The bacteriophage suspension was transferred to a sterile polypropylene tube. Chloroform was added to 4% and then incubated for 15 minutes at room temperature with occasional agitation. Cellular debris and agar were pelleted and the supernatant recovered to sterile glass bottles. Chloroform was added to the phage lysate to 0.3%. The amplified library was aliquoted and stored at 4°C.

II. λ:HUMAN LIBRARY SCREEN

1. Plaque lifts

The λEMBL3:human unamplified library was screened as described by Benton and Davis (1977) with modifications. $7x10^4$ pfu were incubated with 1 ml of NM 539 bacterial culture (per Nunc culture

dish) and plated using 0.7% agarose in TM top medium. With the appearance of plaques following a 10-hour growth period, the plates were cooled to 4°C for 1-2 hours. Dry Biodyne A membranes (Pall) were placed on the plates for 1 minute to adsorb phage DNA. The plates and the membranes were appropriately marked with Indian ink for orientation purposes. The DNA was denatured by placing the filters on 3MM Whatman paper soaked with a solution of 1.5M NaCl and 0.5M NaOH. 3M Na acetate (pH 5.5) was used to neutralize the DNA bound to the filters. The filters were air-dried for approximately 30 minutes and baked under vacuum at 80°C for 2 hours.

2. DNA purification from low melting point agarose gels

Following the procedure of Maniatis *et al.* (1982), the DNA restriction fragments were electrophoresed in a 0.8% low melting point agarose gel (BRL). The fragment of interest was excised with minimum agarose, cut into pieces and dispensed in a Corex tube with 5-10 ml of TE buffer. It was then heated to 65°C until the complete melting of the agarose was achieved. The DNA was phenol-extracted as above.

3. Nick-translation

DNA fragments were nick-translated as described by Maniatis *et al.* (1982). The reaction contained, in a total volume of 50 µl: 1 µg DNA, 50mM Tris.HCl (pH 7.5), 10mM $MgSO_4$, 0.1mM DTT, 50 µg BSA, 20mM dATP, dGTP, TTP, 0.107 µM dCTP (α-^{32}P, 3000 Ci/m mole in Tricine, New England Nuclear), 10 units of *E.coli* DNA Pol I and 0.25ng of calf thymus DNase. Following 1 hour incubation at 14°C, the reaction was stopped by the addition of 1 volume stop solution (20mM EDTA, 2 mg/ml

calf thymus DNA and 0.2% (w/v) SDS). A 0.15% (w/v) solution of BPB was added to 1/10 volume of the mixture. The sample was added to a Sephadex G-50 column, which had been equilibrated with TE and 1mM NaN_3, packed in a disposable syringe and inserted into a plastic tube. After centrifugation, the sample was collected and the specific activity of the radioactive DNA measured by counting a sample of each fraction in distilled water in a Beckman LS7500 liquid scintillation spectrometer.

4. Oligo-priming

As an alternative to nick-translation, oligo-priming enhanced the incorporation of the radioactive nucleotide into the DNA without purification of these fragments from low-melt agarose. The procedure follows that of Feinberg and Vogelstein (1983). The DNA was restricted on a 0.8% low melting point gel and the fragment of interest was excised, weighed, and 1.5 ml of dH_2O added for every gram of gel. After boiling for 7 minutes, an aliquot containing 30-50 ng of DNA to be labelled was incubated at 37°C. The total reaction mixture (volume: 50 μl) had 10 μl of OLB solution (consisting of 100 μl of 1.25M Tris.HCl, 125mM $MgCl_2$ (pH 8), 100mM of β-MeOH, dATP, dGTP and TTP, 250 μl of 2M HEPES (pH 6.6) and 150 μl of hexadeoxyribonucleotides (Pharmacia), suspended in TE), 2 μl of BSA (10mg/ml), DNA to 32 μl, 5 μl dCTP (α-^{32}P, 3000 Ci/m mole, 10 μCi/μl) and 2 units of Klenow fragment of *E.coli* DNA Pol I. After 2-4 hours of incubation at 37°C, the reaction was stopped by the addition of 20mM NaCl, 20mM Tris.HCl (pH 7.5), 2mM EDTA, 0.25% SDS and 1 μM dCTP. The unincorporated radioactive nucleotides were removed by spun-column chromatography and the specific activity of radioactive DNA determined as described above.

5. Hybridization

Nick-translated or oligo-labelled DNA probes were hybridized to DNA immobilized on Biodyne A membranes as described by Klessig and Berry (1983). The pre-hybridization and hybridization solutions contained 50% (v/v) deionized formamide, 500 µg/ml denatured salmon sperm DNA, 1m NaCl, 50mM PIPES (pH 7), 0.5% (w/v) Sarkosyl, 5x Denhardt's solution [0.15% (w/v) each of Ficoll, polyvinylpyrrolidine and BSA], 20 µg/ml yeast tRNA, 10mM EDTA (pH 8) and distilled water to 6% of the total volume. 20 ml of the solution was used to pre-hybridize the baked filters in a 42°C shaker waterbath for 2-4 hours. Hybridizations were carried out in half the volume with the addition of dextran sulphate (10% w/v) and 30-50 ng of dCTP (α-^{32}P) labelled probe, denatured by boiling for 5 minutes in distilled water. The specific activity of the probe varied from 0.5×10^8 to 5×10^8 cpm/µg of DNA. The filters were hybridized at 42°C in a waterbath with gentle agitation for 24-48 hours.

6. Washes

The blots were washed thrice in 2xSSC (300mM NaCl, 30mM Na acetate and 0.1% Sarkosyl) 5-10 minutes each at room temperature, once in 1xSSC, 0.5% Sarkosyl for 30 minutes at 50-53°C and thrice in 0.1xSSC and 0.1% Sarkosyl for 30-60 minutes each at 50-53°C.

7. Autoradiography

Filters were marked with ^{35}S-Indian ink and exposed to Kodak XAR films in x-ray cassettes with intensifying screens (Dupont) at -70°C. Following appropriate exposure periods, the films were

developed manually using Kodak X-ray Developer and Rapid fixer.

III. CHARACTERIZATION OF THE CLONES

1. Southern transfer

Restricted DNA fragments were electrophoresed and blotted to Biodyne A membranes using the procedure of Southern (1975) with modifications. Following electrophoresis, the agarose gel was denatured in a solution of 500mM NaOH and 1.5M Tris (pH 7.5) for 30 minutes with gentle shaking, and neutralized in 3M Na acetate (pH 5.5) for 30 minutes at room temperature. Gels were placed directly on a wick made up of 3MM Whatman filter paper soaked in 20xSSC and overlaid with a piece of dry Biodyne A membrane. Several pieces of 3MM Whatman paper were placed on the filter and overlaid with a 3 inch stack of paper towels and a one kilogram weight. Following 16-24 hours of blotting in 20xSSC, the Biodyne filter was air-dried and baked for 1 hour at 80°C under vacuum. Hybridization of the DNA-bound filters to the desired probe was carried out as described above.

The Southern blot of human genomic DNA was carried out with slight modifications. Biodyne A membranes were replaced with Gene Screen Plus filters (NEN, Dupont) and used to bind DNA. After the electrophoresis of the human DNA, the gel was acid-nicked in 0.25N HCl for 15 minutes at room temperature prior to alkaline treatment (0.4N NaOH, 0.6M NaCl for 30 minutes at room temperature). Following the neutralizing step (as before), the gel was placed on a wick and overlaid with Gene Screen Plus membrane (soaked in distilled water and 10xSSC). Transfer of the DNA onto the filter was allowed to proceed

for 16-24 hours in 10xSSC after which the filter was immersed in an excess of 0.4N NaOH for 30-60 seconds and then rinsed in 0.2M Tris (pH 7.5) and 2xSSC. As there was no requirement for baking, the filter was air-dried and hybridized to DNA probes as before. The radioactive membrane was washed in 2xSSC for 5 minutes, 2xSSC and 1% SDS for 30 minutes at 65°C and finally in 0.1xSSC at room temperature, all with constant agitation.

2. Southern Cross Restriction Mapping

Rudimentary restriction maps of the recovered positive clones were constructed using single and double digests with various restriction endonucleases. Southern Cross Restriction Mapping System (NEN, Dupont) was used to further verify the restriction maps. The principle is based on the homology between different restriction fragments of the same DNA clone. For example, a DNA fragment to be mapped is digested with 3 different enzymes, with DNA from one of the reactions labelled with a radioisotope. Each reaction is individually run on an agarose gel and blotted onto a filter. All membranes are then stacked up on one another. The radioactive fragments (bound to a special filter and laid perpendicular to the other filters) are released under prescribed conditions and hybridize to homologous sequences of other non-radioactive fragments. "Hybridization spots" on autoradiographs of the filters indicate that the 2 fragments either overlap or are contained within one another. Details of the protocol are stated below.

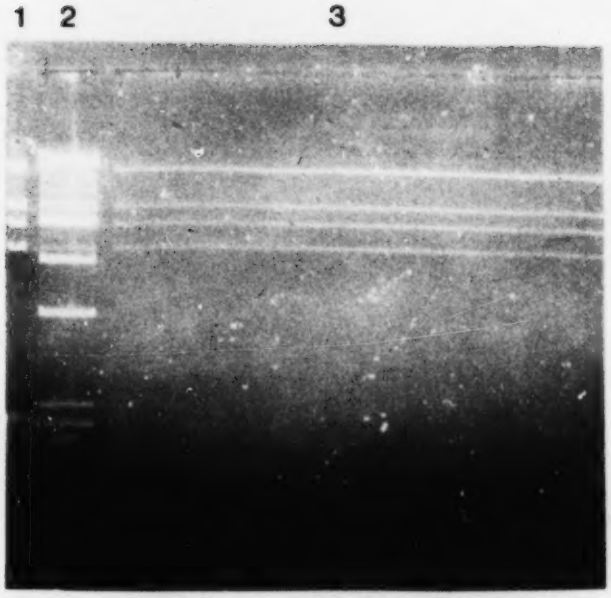
a. Gel electrophoresis and transfer of the non-radioactive digests

DNA digests with different enzymes were individually run on 0.4% agarose gels (22x25 cm: 0.6 cm thick. Apparatus: Tyler Research). The special combs formed a large preparative well for the sample, a small well for analytical purposes and finally one for molecular weight marker DNA. TAE buffer and marker dye were added to 2µg of DNA and the mixture was loaded into the gel slot. One microgram of the DNA sample was used to monitor the migration of the bands in the preparative slot. The voltage was kept low (2 volts/cm) to prevent the distortion of the bands. A sample of λcI 857 *Hind* III digest was used as the molecular weight standard (Fig. 4). After the electrophoresis was complete, the DNA was blotted to Gene Screen Plus membranes (in 25mM Na phosphate buffer, pH 6.8). Prior to the transfer, the gel was acid-hydrolyzed (10-15 minutes) and denatured for 45 minutes (as for a Southern transfer). The gel was incubated in a solution of 3M NaCl and 0.5M Tris.HCl (pH 7) for 60 minutes and blotted for at least 20 hours. The filter was then air-dried and stored at room temperature for a further cross-hybridization experiment with other non-radioactive digests (blotted in the same manner) and the radio-labelled digest.

b. Gel electrophoresis and transfer of the radioactive digest

The restriction digest of choice was end-labelled by filling the recessed 3'-OH end of the double-stranded DNA. The reaction was carried out as recommended by Maniatis *et al.* (1982). As much as 1 µg of DNA was digested with the restriction enzyme of interest. 2 µCi of an appropriate ^{32}P-dNTP (in this case α-^{32}P dATP) and 1 unit of the Klenow fragment of *E.coli* DNA Pol I were added. The mixture was

Figure 4 Southern Cross gel electrophoresis of λ3-B4 *Sal* I/*Bam* HI restriction. Approximately 2 μg of DNA was digested and loaded into the wide slot (3). Electrophoresis was performed for 18 hours at 40 volts, corresponding to 2 V/cm. The smaller sample DNA slot (1) and the λcI857 *Hind* III marker slot (2) were excised before blotting the gel. (*) indicates the λ arms.


1
2
3
-20*
-11.6
- 9*
- 7.4
Kb

incubated at room temperature for 10-30 minutes and the DNA fragments purified by spun-column chromatography. The radio-labelled DNA digest was electrophoresed and blotted as with the non-radioactive digests; however, the acid-hydrolysis step was omitted and the Gene Screen Plus filter replaced with Gene Screen (NEN, Dupont) filter.

c. Cross blot construction

Before the complete transfer of the radioactive digest, all of the non-radioactive transfer membranes (total of 4) were washed in 100 ml of Hybridization Buffer Concentrate (HBC), 100 ml of distilled water and 2 ml of 10% SDS (hybridization buffer A) for several hours at 65°C with gentle agitation. Hybridization buffer C was prepared by mixing the following components: 25 ml HBC, 25 ml dH_2O, 0.5 ml 10% SDS and 0.5 ml 10% (w/v) Na pyrophosphate. 2 non-radioactive transfer membranes to be used adjacent to the radioactive membrane in the cross-blot were washed in the degassed buffer B solution. The remaining unlabelled DNA-bound membranes were washed at room temperature in degassed hybridization buffer C (125 ml HBC, 125 ml deionized formamide and 2.5 ml of 10% SDS). Next, a plastic tray (25x25 cm) was balanced on a small (about 22x22 cm) support and a glass plate was placed in it. A non-radiolabelled transfer membrane was removed from buffer C and placed, DNA-bound side facing up, on the glass surface. Air bubbles were carefully rolled out during this and the subsequent steps. Another membrane from buffer C was placed on the filter in the same orientation . The radioactive membrane was removed from the blotting stack and briefly soaked in degassed 25mM

Na phosphate (pH 6.8). It was then placed, face up, on the stack perpendicular to the orientation of the non-radioactive transfer membranes. One of the membranes from buffer C was placed on the ^{32}P-dATP labelled filter in the same orientation as other unlabelled filters but facing down. The remaining membrane from buffer C was stacked, the DNA-bound side facing the radioactive filter. Finally, a piece of blotting paper was soaked in buffer C and placed on top of the stack. 5-8 ml of the same buffer was spread over the entire stack (with emphasis on the edges of the membranes) and the air-bubbles were removed. Hybridization was carried out for 1-2 hours at room temperature and overnight at 37-42°C.

d. Washes and autoradiography

Following hybridization, the non-radioactive membranes were removed from the stack and washed 3x in 2xSSC, 0.1% SDS at room temperature for 30 minutes with gentle agitation. The filters were then transferred to a solution of 0.1xSSC and washed for 40 minutes, and then air-dried. Exposure period of the blots varied and depended on the position of the membrane in the stack, approximately 4-14 days. The XAR films were developed as described previously.

Northern Transfer

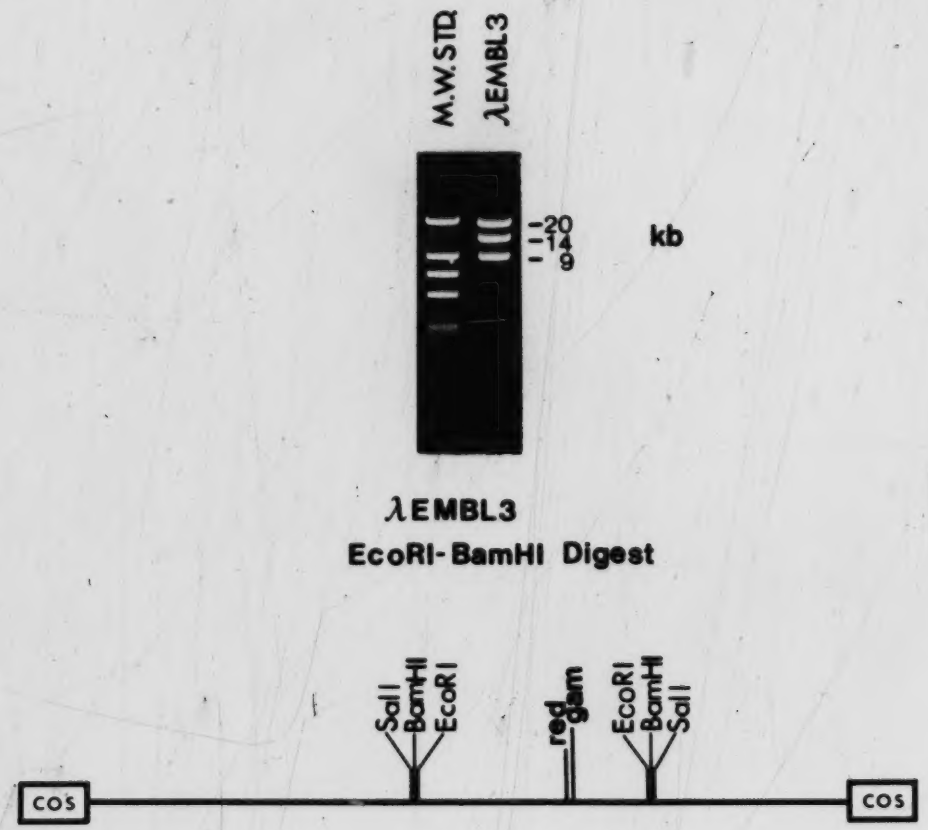
RNA from the human T-lymphoma cell-line Jurkat (treated with PHA, Concanavalin A and cyclosporin) was kindly provided by Dr. J. Elliot. Northern blots, to be reprobed with the human λ3-B4 *Bgl* II fragment, were gifts from Dr. R. D. Gietz.

RESULTS

The identification of the putative lipoxygenase clones

The use of bacteriophage λEMBL3 as the vector for the construction of the λ:human library provided distinct advantages over the other cloning vector systems. λEMBL3 vectors have a large capacity [9-22 kilobases (kb)] and a polylinker sequence with unique restriction sites flanking the central fragment. Figure 5 shows the organization of the λEMBL3 DNA. Due to the *spi*$^+$ (sensitive to P2 infection) phenotype of the wild type, conferred by *red* and *gam* gene products, this phage cannot grow on the P2 lysogenic *E.coli* host strain. Since the two genes lie on the central "stuffer" fragment of the phage, replacement of these fragments with inserts would result in *spi*$^-$ phenotype. Therefore, only the recombinant phage particles can grow on the restrictive host (P2 lysogen NM539 *E.coli* strain). Moreover, the *Eco* RI/*Bam* HI double digests at the polylinker sequences prevent the ligation of the stuffer fragment to the arms and hence eliminate the requirement for the purification of the arms (Frischauf *et al.*, 1983). To ensure the representation of approximately 99% of the human genomic DNA, at least 8×10^5 pfus were obtained. This is assuming that the human Sau 3A fragments averaged about 17 kb in size. For large scale ligations and packagings, the ratio of 2 (arms):1 (insert) yielded about 4×10^5 pfus per single packaging extract. However, a total of 1.2×10^6 pfu from the unamplified library were screened (as described in Materials and Methods). For statistical purposes, the use of the unamplified library to isolate the human lipoxygenase gene was preferred over the

Figure 5 The restriction map of λEMBL3: 15-22 kb human DNA fragments were ligated into the *Bam* HI site following the double-digestion of the phage DNA with *Eco* RI and *Bam* HI restriction enzymes (Frischauf *et al.*, 1983). Bands labelled 20 kb and 9 kb represent the left arm and the right arm, respectively. The 14 kb stuffer region, containing *red* and *gam* genes, is removed to allow for insertion of foreign DNA.


M.W.STD
λEMBL3
20
14
9
kb
λEMBL3
EcoRI-BamHI Digest
Sal I
BamHI
EcoRI
red
gam
EcoRI
BamHI
Sal I
cos
cos

amplified. The latter was aliquoted and stored at 4°C for future use.

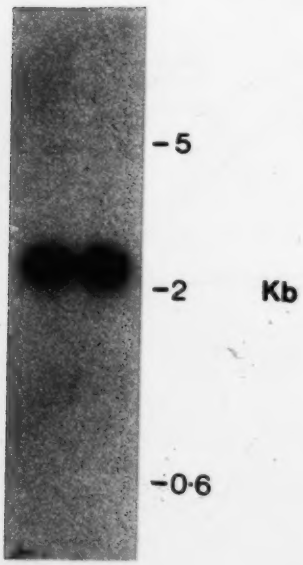
Rabbit Lipoxygenase Probe : A one-kilobase rabbit lipoxygenase cDNA fragment, kindly provided by Dr. R. D. Gietz, was used as the probe. Fractionated rabbit reticulocyte mRNA (derived from rabbits made severely anaemic by phenylhydrazine injections) was utilized as the template for the cDNA synthesis of the lipoxygenase fragment (Okayama and Berg, 1982). As proof of identity, the cloned cDNA was able to select the rabbit reticulocyte lipoxygenase mRNA from the total RNA pool. The cDNA fragment, bound to nitrocellulose, was hybridized to mRNA in solution and the filter extensively washed. Subsequently, the mRNA was released from the hybrid and translated in a cell-free system (reticulocyte lysate) as described by Maniatis *et al*. (1982). The resultant protein had identical migration properties as that of pure reticulocyte lipoxygenase on SDS polyacrylamide gels (Fig. 6). The cDNA was cloned in a pUC8 plasmid derivative, pSV88. Northern blot analysis of the poly $(A)^+$ mRNA was carried out using the lipoxygenase cDNA (MR7-*Xba*) fragment. The size of the transcript was estimated at 2.5 kb (Fig. 7).

Following the initial screening of the λ:human library with the MR7 *Xba* fragment, three putative lipoxygenase clones were identified. Two cycles of rescreening verified the homology of these recombinants to the rabbit lipoxygenase cDNA. Lysates obtained from single plaques were used to isolate DNA from these recombinant phages. *Sal* I endonuclease restriction of these clones, henceforth designated as λ2-A1, λ2-41, and λ3-B4, was carried out as described in Materials and Methods. This enzyme cleaves the λDNA at polylinker sites flanking the middle fragment, thereby excising the entire human DNA clone.

Figure 6 Positive mRNA selection by rabbit reticulocyte lipoxygenase cDNA. The identities of a number of cDNA clones were determined by *in vitro* translation of hybridization-selected complementary mRNA. Clones MR7 and MR9 contain cDNA inserts coding for the lipoxygenase gene. The blank lane represents the translation products of transcripts endogenous to the rabbit reticulocyte lysate. Translational products from total mRNA fraction are depicted in the mRNA lane. The hybrid-selection of mRNA complementary to a different population of cDNA, while serving as an important positive control for the selection procedure, is not shown in this autoradiograph (Figure kindly contributed by Dr. R. D. Gietz).

mRNA
Blank
MR4
MR7
Lipoxygenase

Figure 7 Northern blot analysis of lipoxygenase mRNA. 2 μg of rabbit reticulocyte polyadenylated mRNA was electrophoresed on a formaldehyde gel and blotted to nitrocellulose, then probed with the MR7 cDNA insert. The lipoxygenase mRNA is approximately 2.5 kb in length (Figure generously contributed by Dr. R. D. Gietz.)


-5
-2
Kb
-0·6

Inserts from λ2-A1 and λ2-41 seem to have an internal *Sal* I site each whereas the human DNA from λ3-B4 was excised as a single fragment. The size of the sequences cloned varied from 17 kb (λ2-A1) to 22 kb (λ2-41). Using λcI857- *Hind* III as a molecular weight standard, the size of the λ3-B4 insert was calculated to be approximately 19 kb.

Restriction map

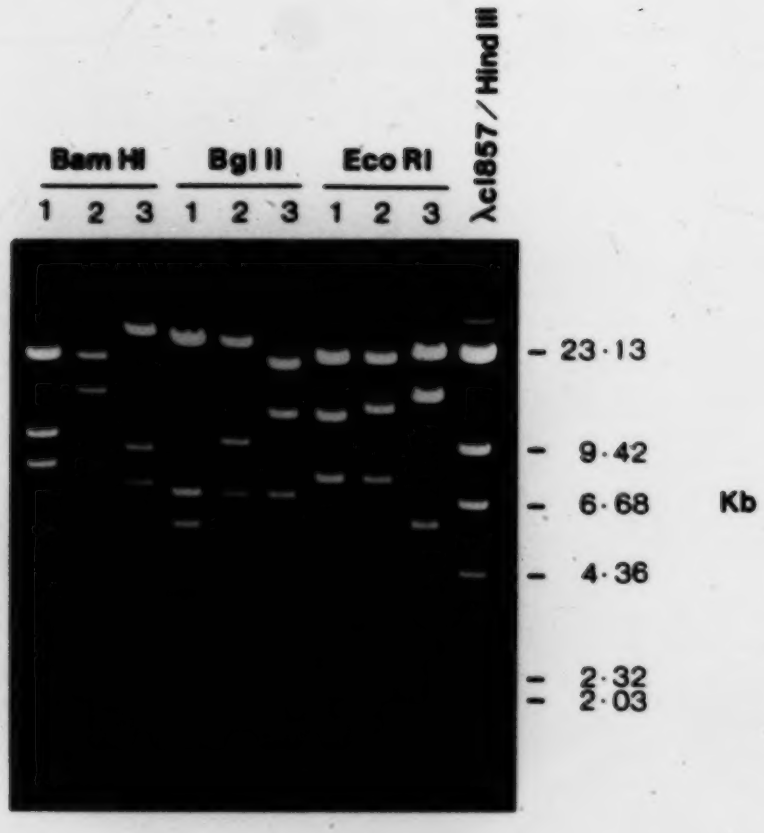
The first step to elucidate the structural organization of the cloned human DNA sequences homologous to the rabbit lipoxygenase cDNA was to construct a detailed restriction map.

Restriction sites were mapped by analysis of DNA fragments produced by single- or double-digestions with various enzymes. However, to map a region which spanned almost 30 kb of the human genome proved to be both tedious and time-consuming. Therefore, an alternative method, known as Southern Cross Mapping System, was employed which eliminated the necessity for subcloning. Initial observations from *Bam* HI, *Bgl* II and *Eco* RI digests of the λ clones showed that λ2-A1 and λ2-41 carried DNA sequences that overlapped extensively. λ3-B4 insert overlapped to a lesser degree (Fig. 8). After restriction digestion with several other endonucleases, a rudimentory map was established.

Analysis of the Southern Cross Hybridizations

By using the Southern Cross Mapping System, the order of certain ambiguous sites on the map was clarified. Since a large region of λ2-A1 overlapped with λ2-41, only the latter and λ3-B4 were selected for the experiment.

Figure 8 Restriction digest analysis of λ2-A1 (1), λ2-41 (2) and λ3-B4 (3). Approximately 1 μg of each DNA sample was digested with specific enzymes and electrophoresed on a 0.4% agarose gel. λcI857 digested with *Hind* III serves as a size marker. Comigrating bands in distinct clones digested with identical restriction endonucleases represent homologous sequences and provide evidence for extensive overlap of the three human genomic lipoxygenase clones.


Bam HI
Bgl II
Eco RI
λcI857 / Hind III
1 2 3 1 2 3 1 2 3
23·13
9·42
6·68
4·36
2·32
2·03
Kb

Following *Sal* I digestion and purification from low-melt agarose, the inserts were cleaved by *Bam* HI, *Bgl* II, *Eco* RI & *Hind* III enzymes. The exact size of each fragment was determined. Care was used to avoid doublets that could not be resolved easily. Gel electrophoresis and transfer of each non-radioactive digest to Gene Screen Plus membrane was performed as described in Materials and Methods. These DNA fragments were permanently bound to the filters. *Sal* I fragments of λ2-41 and λ3-B4 were digested individually with *Eco* RI, end-labelled and electrophoresed as described earlier (Note: Non-radioactive *Eco* RI digests were used as positive controls for the hybridization procedure). Labelled fragments were transferred to Gene Screen membranes which allowed the DNA to be eluted under prescribed conditions and hence, to serve as hybridization probes. To detect homologous sequences, Gene Screen Plus membranes with non-radioactive fragments were stacked over the Gene Screen filter (containing the labelled fragments) at a 90° angle. Under conditions which promoted both the release of radioactive fragments from the Gene Screen membrane, and hybridization of homologous sequences, labelled DNA diffused through the layered membranes. Following washing and the autoradiography of the filters, labelled fragments which had "located" homologous sequences in the radioactive samples appeared as "hybridization spots" (Figs. 9 and 10). Only representative autoradiographs are shown in this text. Clearly, the hybridization of radioactive *Eco* RI fragment (λ3-B4) to the unlabelled λ3-B4 *Sal* I/ *Eco* RI fragments would produce a diagonal array of spots (Fig. 9). The methodology to work out the sequence of restriction sites is schematically presented in Fig. 11, based on the data from

Figure 9 Southern Cross analysis of λ3B-4 *Sal* I/*Eco* RI digest. Autoradiograph of the ^{32}P-dATP *Eco* RI digest of λ3-B4 *Sal* I fragment (I), cross-blotted to the unlabelled λ3-B4 *Sal* I / *Eco* RI fragments (II). Arrows indicate the direction of DNA migration. 20* and 9* kb fragments represent λ left arm and right arm, respectively. The linear array of hybridization spots is indicative of absolute homology between the cold DNA and radiolabelled probe in this instance.

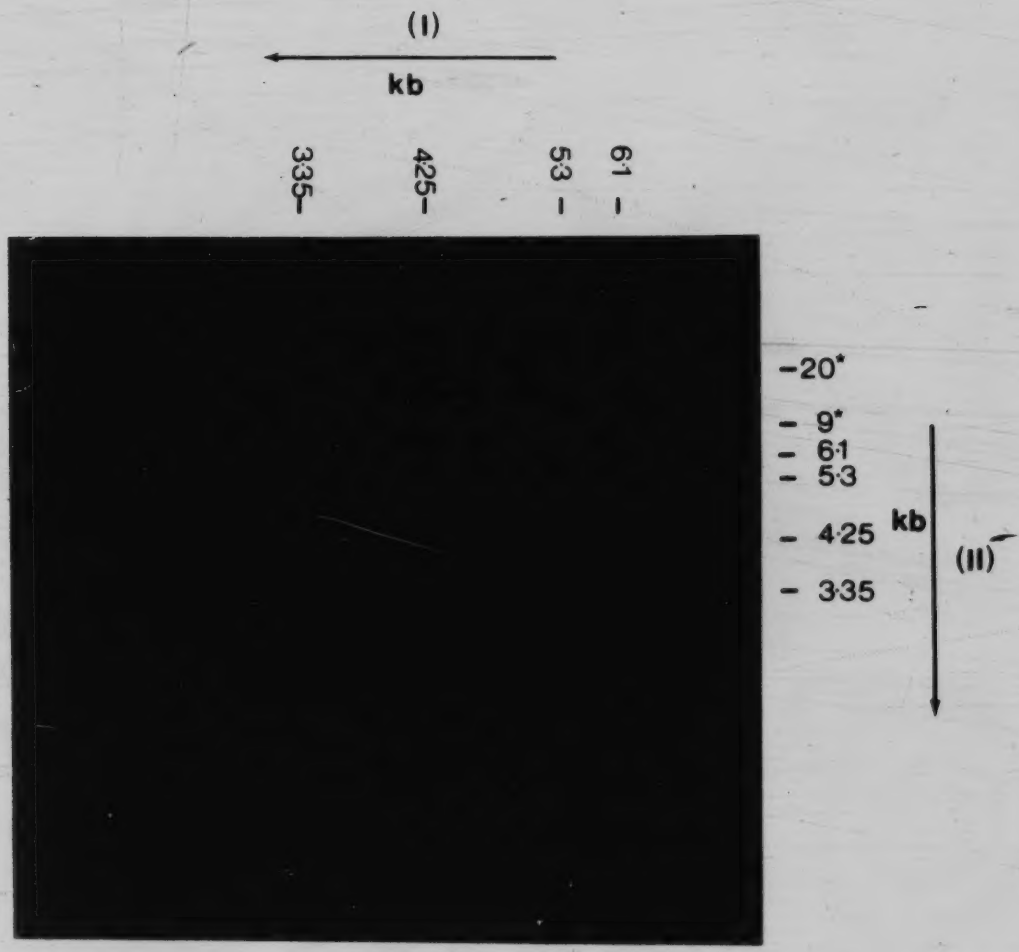

(I)
kb
6·1
5·3
4·25
3·35
20*
9*
6·1
5·3
4·25
3·35
kb
(II)

Figure 10 Southern Cross analysis of λ3-B4 *Eco* RI and λ3-B4 *Bam* HI digest. Autoradiograph of the Southern Cross hybridization of λ3-B4 *Eco* R digestion of *Sal* I fragment (^{32}P-labelled)(I) with λ3-B4 *Sal* I *Bam* HI digest (II). The arrows indicate the direction of migration of DNA on the agarose gels. λarms are marked by (*). The interpretation of this autoradiogram is provided in the text and in Figure 11.

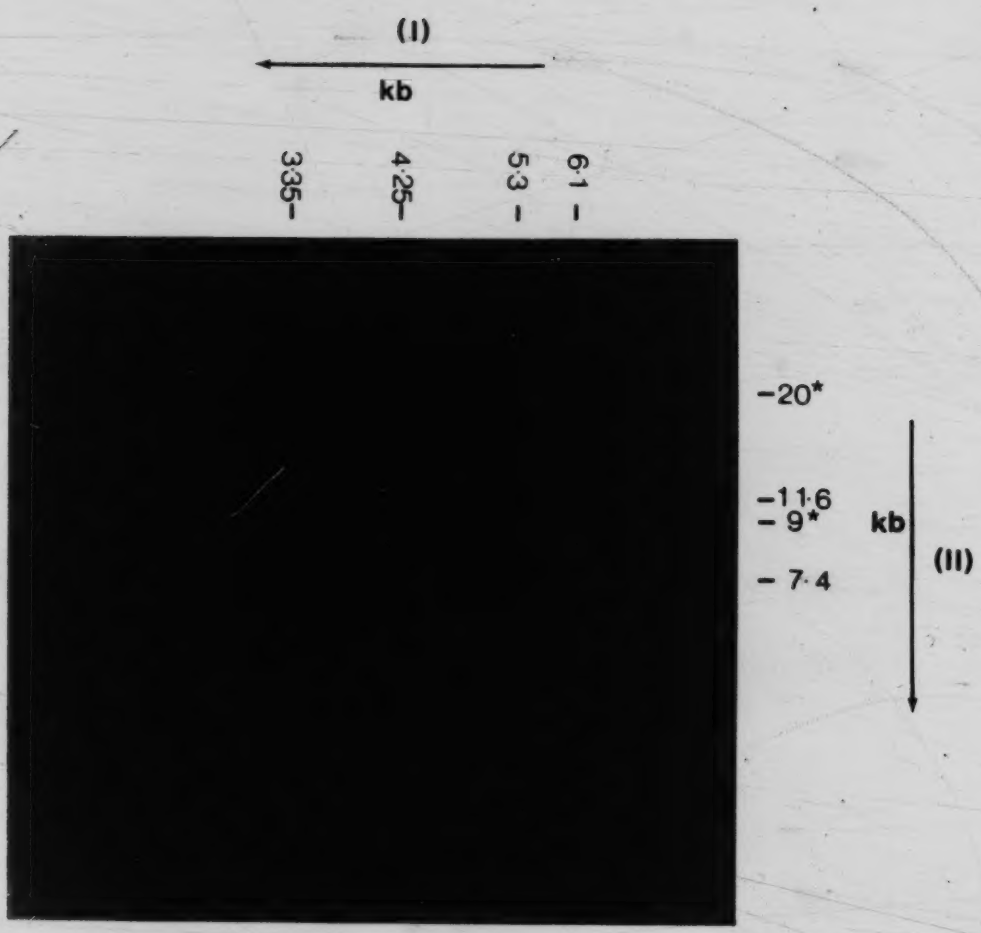
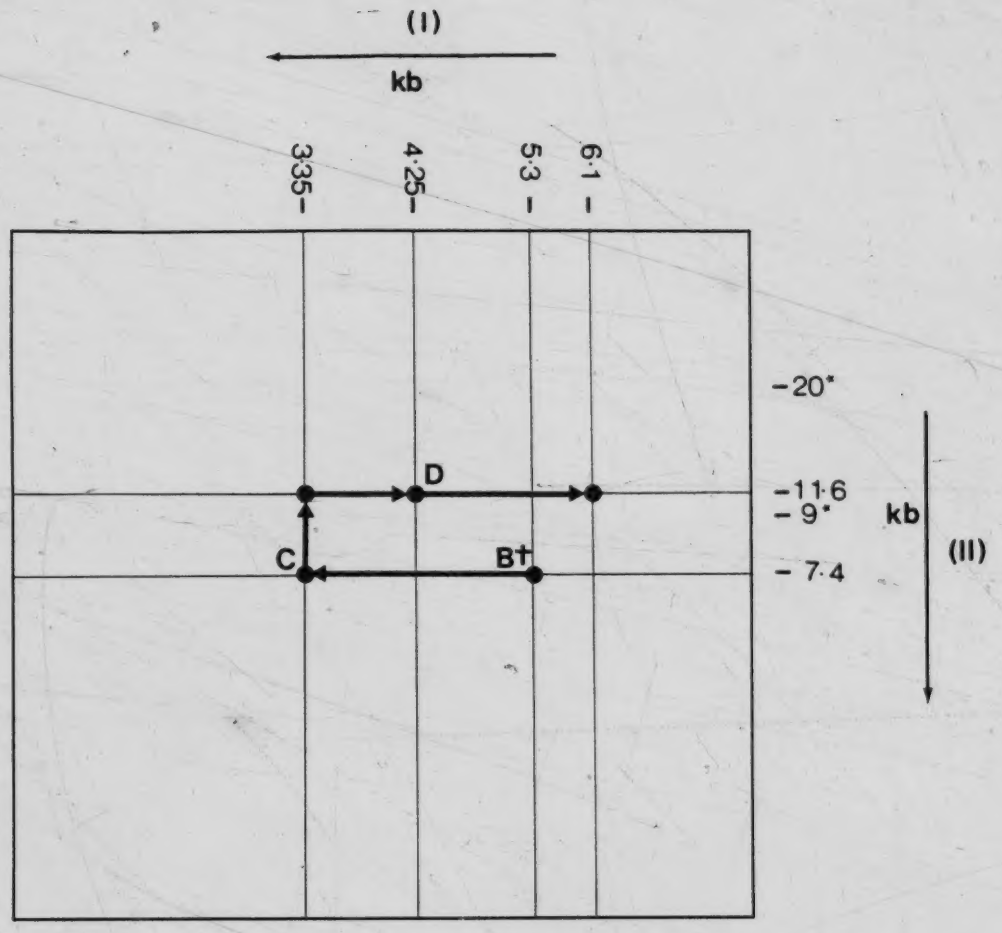
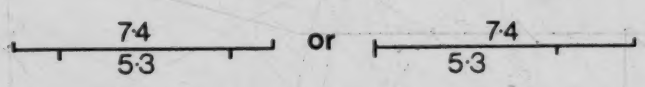

(I)
kb
6·1
5·3
4·25
3·35
20*
11·6
9*
7·4
kb
(II)

Figure 11 Schematic interpretation of Southern Cross Mapping. The ^{32}P-dATP end-labelled of *Eco* RI digest of λ3-B4 *Sal* I fragment (I), cross-blotted to non-radioactive λ3-B4 *Sal* I *Bam* HI fragment (II). Arrows indicate the direction of DNA migration and (*), the λ arms. (†) marks the starting point that can be arbitrarily assigned; this "hybridization spot" signifies the cross homology between the 5.3 kb *Eco* RI fragment and the 7.4 *Bam* HI fragment. Since the former is not homologous to any other *Bam* fragment, it is contained entirely within the 7.4 kb *Bam* HI fragment. These spots can be covered vertically and horizontally. In this case, the choices made depended on the map position of of the restriction fragments with respect to the λ arms. See Figure 10 for autoradiogaph.

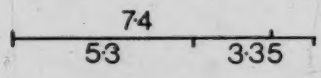


A

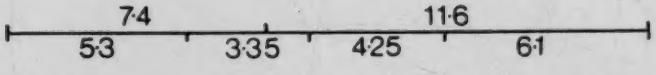
B Bam HI / Eco RI



C



D



or

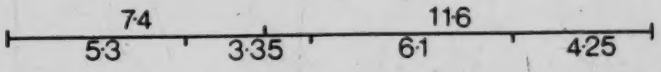


Fig. 10. The starting point has been indicated by (†). This spot represents hybridization between the 5.3 kb *Eco* RI fragment (E-5.3) and the 7.4 kb *Bam* HI fragment (B-7.4). Since E-5.3 does not hybridize to any other *Bam* HI fragment, it seems to be contained entirely within B-7.4. Figure 11 (B-D) shows the sequential construction of λ3-B4 *Eco* RI and *Bam* HI maps relative to one another as all spots on the autoradiograph are covered in the direction of the arrows.

The detailed restriction maps of λ2-A1, λ2-41 and λ3-B4 are illustrated in Figure 12.

Characterization of the transcriptional unit(s)

The identification of coding regions in the three human lipoxygenase clones was carried out. λ2-A1, λ2-41 and λ3-B4 were all digested with different restriction endonucleases, electrophoresed and transferred to Biodyne membranes (as described in Materials and Methods). The one-kilobase rabbit lipoxygenase cDNA fragment was used to probe the filters. Figure 13 depicts the homology between the rabbit lipoxygenase fragment and the putative human lipoxygenase sequences. It is of interest to note that certain bands [13 kb λ2-41 *Eco* RI fragment (1) and 6.1 kb λ3-B4 *Eco* RI fragments (2)] seemed to share less homology with the probe. The significance of this point is discussed later. The final analysis of several Southern transfers concluded that an 8.5 kb region of λ3-B4 and λ3-41, and a 5.5 kb sequence of λ2-A1 hybridized to the MR7-*Xba* I fragment (Fig. 12).

Human genomic Southern analysis

Human and rabbit genomic DNA were digested with *Bam* HI,

Figure 12 Restriction maps of λ2-A1, λ2-41 and λ3-B4. Hatched box represents the human lipoxygenase coding region homologous to the rabbit cDNA fragment. In λ3-B4, a *Bam* HI site, unique to the clone, is depicted at the junction of the right arm and the insert. The presence of the restriction site could be due to the reconstruction of the *Bam* HI recognition sequence at the polylinker site or a polymorphism.

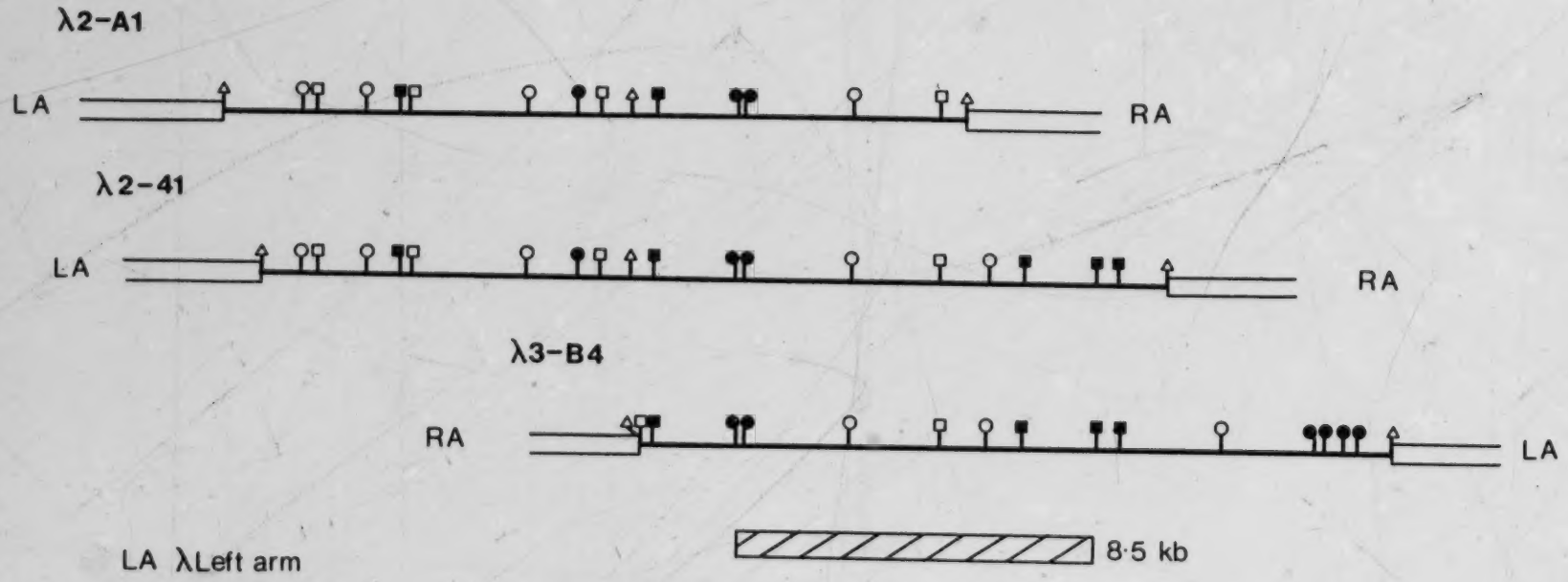
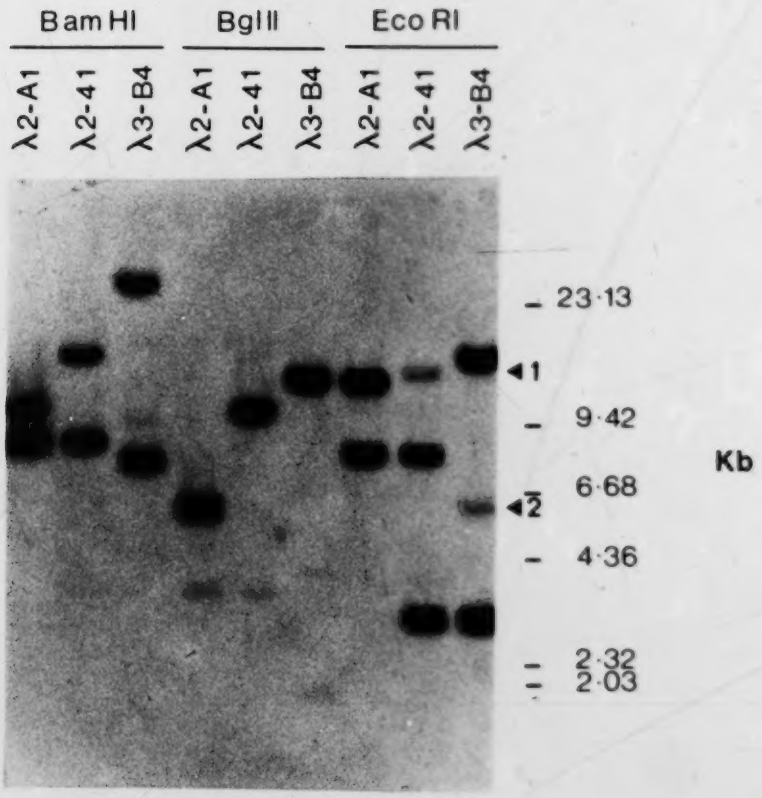

λ2-A1
LA
RA
λ2-41
LA
RA
λ3-B4
RA
LA
8·5 kb
LA λLeft arm
RA λRight arm
BamHI
Bgl II
Eco RI
Hind III
Sal I
1 kb

Figure 13 Southern blot analysis of the three human lipoxygenase clones. 1 μg of DNA was restricted, electrophoresed on a 0.4% agarose gel and blotted to Biodyne membrane. MR7- *Xba* fragment was used to probe the filter. Bands 1 and 2 are fragments that share less homology to the probe signifying the possible presence of other sequences in the genome that are related to the reticulocyte lipoxygenase. The weak hybridization signal can also relate to non-coding regions of the lipoxygenase clones. Molecular size markers were generated by *Hind* III digestion of bacteriophage λ.


BamHI
BglII
EcoRI
λ2-A1
λ2-41
λ3-B4
λ2-A1
λ2-41
λ3-B4
λ2-A1
λ2-41
λ3-B4
23·13
1
9·42
6·68
2
4·36
2·32
2·03
Kb

Bgl II, *Eco* RI and *Hind* III restriction endonucleases. The hybridization of the human DNA sequences to the MR7-*Xba* I fragment was compared to that of the cloned human DNA inserts (Table 2). The purpose was to determine whether any DNA rearrangements had occurred during the course of the human lipoxygenase gene cloning. Several genomic DNA fragments that hybridized to the cDNA probe (Fig. 14) seemed to correspond to the cloned human DNA sequences in λEMBL3 (Fig. 13). For example, a human genomic 8 kb *Bam* HI fragment was equivalent in size to the 8 kb fragment from λ2-A1 and λ2-41 *Bam* HI digest that hybridized to the probe. In the *Bgl* II digest of human DNA, a major band of 14 kb was detected which appeared to be the same as the 14 kb *Bgl* II band of the recombinant phage λ3-B4. In the *Eco* RI digest of the genomic DNA, the 3.35 kb sequence appeared to be identical to an *Eco* RI fragment common to both λ2-41 and λ3-B4. However, certain discrepancies prevailed. Appearance of a few bands, unaccounted for, could relate to restriction sites that were located outside of the cloned region, as in the case of a 15 kb genomic *Bam* HI fragment (Fig. 15, 1). Furthermore, the 10.5 and 6.4 kb *Bgl* II fragments of the human DNA samples were bands that interestingly seemed to share less homology with the MR7-*Xba* I probe than the 14 kb *Bgl* II fragment. Similarly, the signal for the human 5.8 kb *Bam* HI fragment is not as strong as for 8 kb *Bam* HI band. The rabbit genomic DNA fragments seem to have the same characteristics. The interpretations and the implications of these results are discussed later.

Figure 14 Southern blot analysis of human and rabbit genomic DNA. 5 μg of DNA was digested with restriction endonucleases, electrophoresed on a 0.4% agarose gel and blotted to Gene Screen Plus membrane. The probe was the 1 kb rabbit reticulocyte cDNA fragment. Strong hybridization signals such as the human 10 kb *Bam* HI, 15 kb *Bgl* II, and 3.35 kb *Eco* RI bands correspond to identical fragments from the Southern blots of cloned human DNA fragments. The weak signals, on the other hand, are noted fragments that share less homology with the cDNA due to either the presence of large regions that are not transcribed or sequences that are distantly related to the reticulocyte lipoxygenase cDNA.

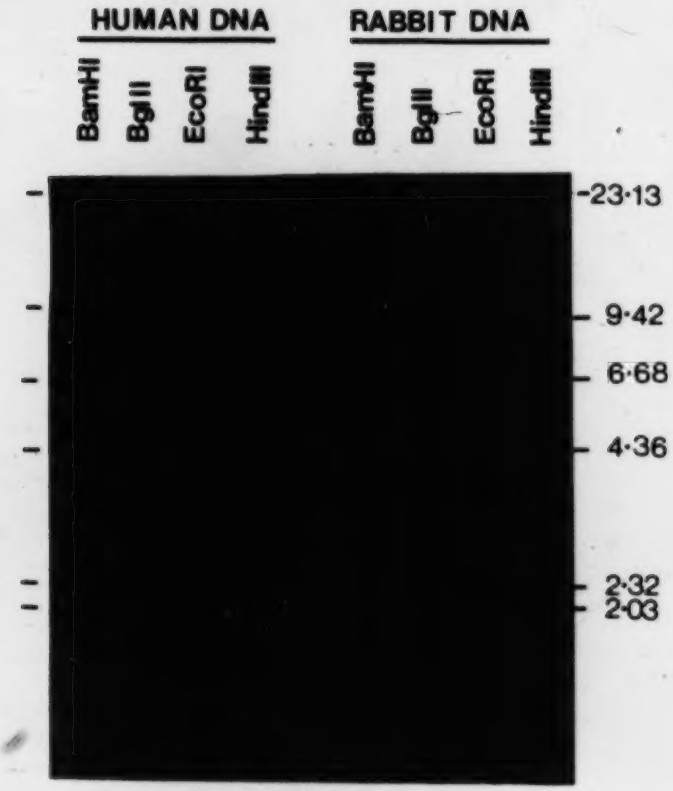

HUMAN DNA
RABBIT DNA
BamHI
BglII
EcoRI
HindIII
BamHI
BglII
EcoRI
HindIII
23·13
9·42
6·68
4·36
2·32
2·03

Figure 15 Genomic restriction map of human lipoxygenase. illustrating the human DNA fragments (————) that hybridize to the rabbit lipoxygenase cDNA Only relevant restriction sites are shown. The verticle lines delimit the region of the genome that has been cloned in λEMBL3. Fragments 1-5 are further elaborated upon in the Southern transfers of genomic and cloned DNA (Table 2).

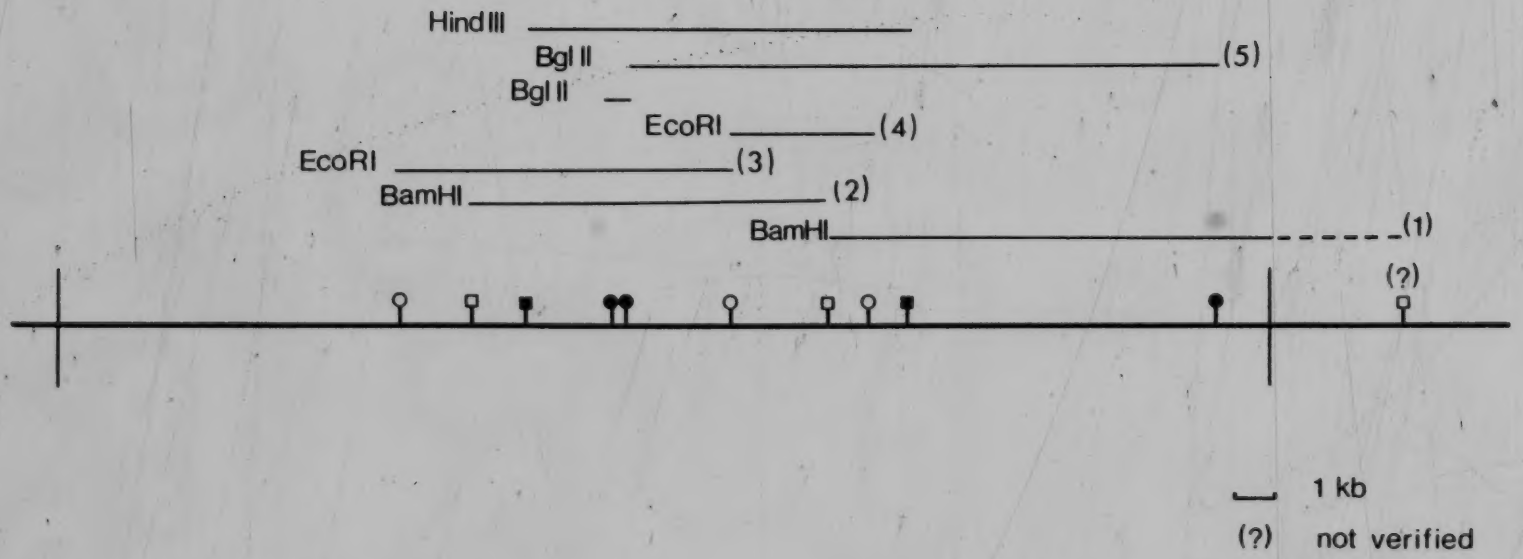

Hind III
Bgl II
(5)
Bgl II
EcoRI
(4)
EcoRI
(3)
BamHI
(2)
BamHI
(1)
(?)
1 kb
(?) not verified

TABLE 2 Comparison of the hybridization pattern between Southern transfers of genomic and cloned human DNA[a].

Size of the restriction fragment	Genomic DNA	Cloned DNA
15 kb *Bam* HI	weak[b]	ND
8 kb *Bam* HI (1)[c]	strong	strong
5.8 kb *Bam* HI (2)	weak	ND
7.5 kb *Eco* RI (3)	weak	strong
3.35 kb *Eco* RI (4)	strong	strong
14 kb *Bgl* II (5)	strong	strong
10.5 kb *Bgl* II	weak	strong[d]
6.4 kb *Bgl* II	weak	strong[d]

a: Both Southern blots were probed with the one-kilobase rabbit reticulocyte lipoxygenase cDNA.

b: Refers to the radioactive signal i.e. the degree of homology between the filter-bound fragments and the probe.

c: Numbers in brackets relate to specific fragments depicted in the restriction map of lipoxygenase region of human genome (Fig. 15).

d: These two bands from λ2-A1 and λ2-41, shown in the Southern transfer of the cloned DNA (Fig. 13) represent sequences that extend into a *Bgl* II site at the 5' end of the λ right arm and hence are not identical to the genomic 10.5 kb and 6.4 kb *Bgl* II fragments.

ND: Not detected

Northern blot analysis

A 3.35 kb *Eco* RI fragment (Fig. 16) that hybridized to the rabbit cDNA was oligo-primed and employed to detect lipoxygenase transcripts in poly(A)$^+$ mRNA from rabbit reticulocytes, human reticulocytes and RNA from the T-lymphoma cell line Jurkat. Hybridization to specific mRNA appeared as a requirement to verify the identity of these putative human lipoxygenase clones.

The scarcity of the human reticulocyte cells (from anaemic blood) and hence the mRNA, proved to be the major difficulty in analysing the transcripts of the gene in question. All RNA isolation, gel electrophoresis and Northern transfers were carried out by Dr. R. D. Gietz and the results from his research were kindly donated to be incorporated into this work.

After purification procedures (see Gietz, 1984), a total of <10 μg of poly(A)$^+$ mRNA from human reticulocytes was obtained and subjected to formaldehyde gel electrophoresis and subsequent Northern transfers. Figure 17 shows the autoradiograph of the Northern blot probed with MR7-*Xba* I fragment. Rabbit reticulocyte mRNA was included as the positive control (lane 1). Evidently, there is strong homology between the lipoxygenase cDNA probe and a 2.5 kb transcript. In the same lane, accumulation of RNA degradation products can explain the appearance of smaller transcripts. RNA from the human lymphoma cell-line was run in lane 2 to study the presence (or absence) of the lipoxygenase message in cells other than reticulocytes. Finally, < 5 μg of human reticulocyte poly(A)$^+$ mRNA was loaded in lane 3. A 2.5 kb mRNA, corresponding to what has been shown to be the rabbit reticulocyte lipoxygenase transcript, was observed.

Figure 16 Cloned human lipoxygenase fragments used to reprobe the Northern transfers. (a) 300 bp *Bgl* II fragment common to all 3 inserts. (b) 3.35 kb *Eco* RI fragment from λ2-41 and λ3-B4. Only the relevant restriction sites on the λ3-B4 map are shown.

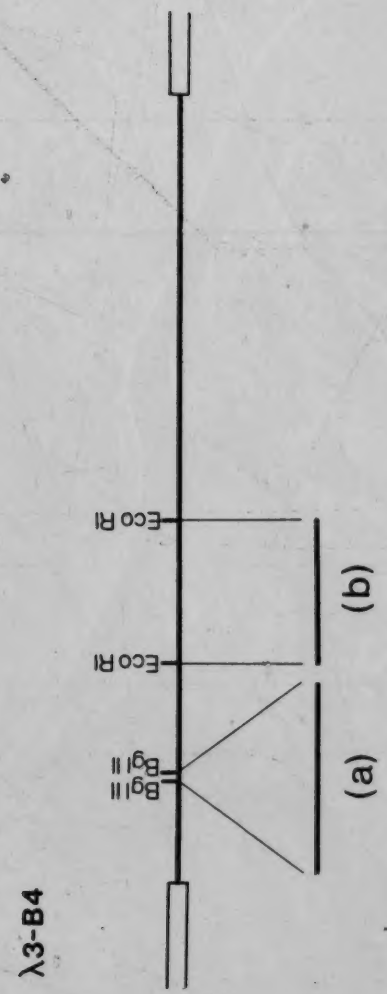
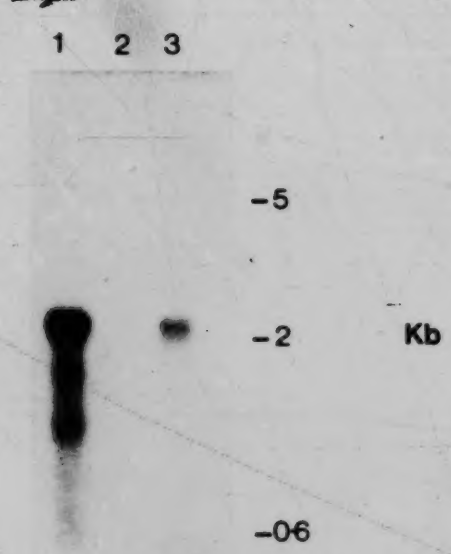

λ3-B4
Bgl II
Bgl II
Eco RI
Eco RI
(a)
(b)

Figure 17 Northern blot #1: 2-4 μg of RNA from rabbit reticulocytes (lane 1), the human T-lymphoma cell-line Jurkat (lane 2) and the human reticulocyte poly(A)$^+$ RNA were electrophoresed on a 1.5% agarose formaldehyde gel, blotted to nitrocellulose and probed with the rabbit reticulocyte lipoxygenase cDNA. Smaller transcripts in lane 1 are likely the result of the breakdown of full length mRNA. The blot was the generous gift of Dr. R. D. Gietz.


1 2 3
-5
-2
Kb
-06

The 3.35 kb *Eco* RI fragment was utilized to probe the second Northern blot (Fig. 18). As with the Northern transfer #1 in Figure 17, the presence of the 2.5 kb transcript was confirmed in rabbit and human reticulocyte mRNA samples (lanes 1 and 3, respectively). However, the 3.35 kb *Eco* RI fragment displayed an apparent "non-specific" binding of the RNA from the cell line. A possible explanation would be the presence of a repeat element in this probe. In an effort to shed light on this matter, the genomic Southern blot was stripped of the MR7-*Xba* I probe and rescreened with the human DNA fragment.

Reprobing the genomic Southern with the 3.35 kb *Eco* RI fragment

The rabbit cDNA probe was removed from the genomic Southern blot (Fig. 14), using 1/20 dilution of the Strip buffer (50mM Tris.HCl pH 8, 2mM EDTA, 1xDenhardts' solution and 0.5% Na pyrophosphate) in a 68°C waterbath for 2 hours with gentle agitation and one solution change. The blot was exposed to X-ray film to ensure the complete removal of the fragment. After prehybridization, the filter was hybridized to the human 3.35 *Eco* RI oligo-labelled fragment for 12 hours. Following the washes (as described previously), the blot was re-exposed to X-ray film at -70°C and developed after 24 hours. Figure 19 lanes H1-4 indicate the hybridization pattern of the human genomic DNA to the probe. Undoubtedly, the 3.35 kb *Eco* RI fragment contained sequences from a repeat element which were absent in rabbit DNA (Fig. 19 lanes R1-4). The presence of repetitive sequences specific to the human genome, such as the *Alu* family, can explain the result obtained.

Figure 18 Northern blot #2: The autoradiograph depicting the hybridization of λ3-B4 3.35 kb *Eco* RI fragment to rabbit (lane 1), human T-lymphoma cell-line Jurkat(2) and reticulocyte (3) polt(A)$^+$ RNA. A 2.5 kb band, corresponding to the size of the lipoxygenase mRNA (Thiele *et al.*, 1982), is detected in human and rabbit reticulocyte RNA fractions. The presence of a repeat element in the probe is the most likely explanation for the selection of the total RNA fraction in lane 2. Since reticulocytes are enucleated and contain only fully processed transcripts, there is no specific homology between the probe and the population of mRNA from these cells. The RNA blot was the kind gift of Dr. R. D. Gietz.

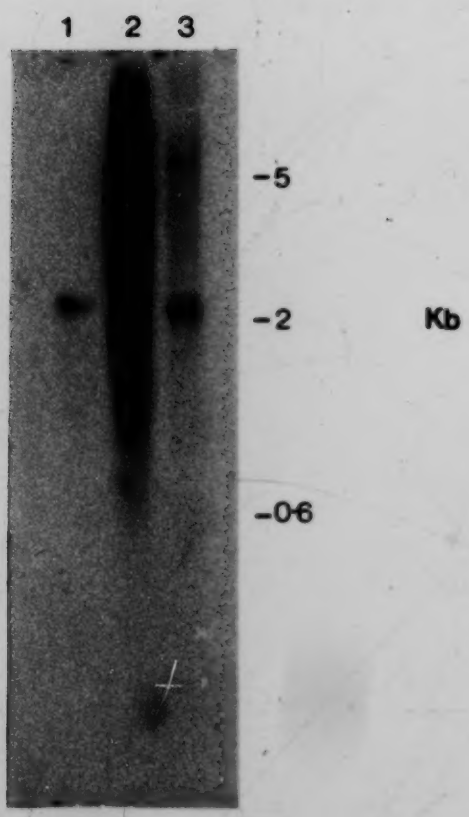
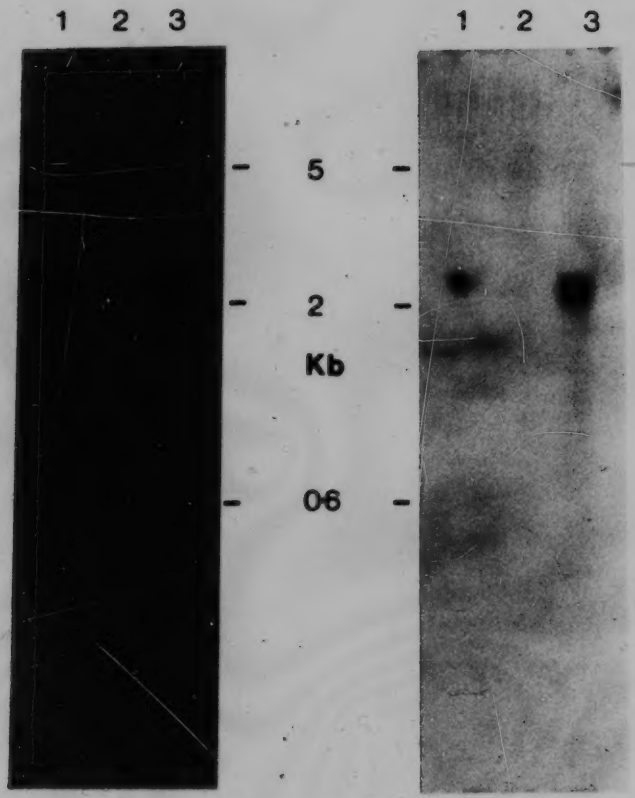

1
2
3
-5
-2
Kb
-06

Figure 19 Genomic Southern blot of human and rabbit DNA, reprobed with λ3-B4 3.35 kb *Eco* RI fragment. The previous probe, MR7-*Xba* I fragment, was removed by the "strip" buffer at 68°C. (Lane 1: *Bam* HI, lane 2: *Bgl* II, lane 3: *Eco* RI and lane 4: *Hind* III digestion). The apparent non-specific binding of the probe to the human DNA is due to the homology between a repetitive element in the human DNA fragment used as the probe and corresponding genomic sequences. Since the repeat element is unique to the human DNA, the presence of an *Alu* repetitive sequence is proposed.

Human DNA
1 2 3 4
Rabbit DNA
1 2 3 4

Figure 20 Hybridization of the human *Bgl* II fragment to Northerns 1 and 2. Following the removal of the MR7 radioactive fragment from the two blots, λ3-B4 300 bp *Bgl* II fragment was used to reprobe the filters (A and B, respectively). The presence of the 2.5 kb lipoxygenase mRNA was confirmed in RNA fractions from both rabbit and human reticulocytes (Lane 1, A and B; lane 3, A and B, respectively). The probe did not show any homology to the T-lymphoma cell-line RNA since it was specifically selected to be devoid of the repetitive element found in the 3.35 kb *Eco* RI fragment.



(A) (B)

Screening of Northern blots with a human *Bgl* II fragment

In the light of the evidence from Northern #2 (Fig. 18) and the genomic Southern (Fig. 19), it was concluded that the 3.35 *Eco* RI fragment contained sequences from a repeat element. Attempts to purify another human DNA probe proved successful. A 300 base pair *Bgl* II fragment (Fig. 16) that hybridized to the MR7-*Xba* I fragment but not to the 3.35 kb fragment was selected as the probe. Northern blots #1 and #2 were stripped of the MR7-*Xba* I fragment and the 3.35 kb *Eco* RI probe, respectively (as above) and hybridized to the *Bgl* II fragment. After a 20-hour exposure period, the films were developed (Fig. 20, A and B). The size of the two transcripts in lanes 1 and 3 once again corresponded to that of the lipoxygenase mRNA. The absence of hybridizing transcript from the cell line RNA was noted and is elaborated upon in the forthcoming discussion.

DISCUSSION

Characterization of the human lipoxygenase clones

Three λclones carrying sequences homologous to a rabbit reticulocyte lipoxygenase cDNA have been isolated from a human genomic library and the inserts mapped using various restriction endonucleases as well as a novel Southern Cross Hybridization technique. This method of mapping proved to be very useful since several discrepancies relating to the order of the restriction sites were resolved in a short period of time.Extensive overlapping regions have been detected in λ2-A1, λ2-41 and λ3-B4. It is therefore proposed that all three inserts span the same region of the human genome. The coding "sequences" have been partially identified using the cDNA as the lipoxygenase probe; however, this fragment does not represent the full-length cDNA of the lipoxygenase message. The 5' and the 3' ends of the fragment have not been determined, and it is therefore not feasible to assign the transcription unit(s) identified to a particular end of the gene. On the other hand, the presence of 8-10 kb on either side of the coding region raises the possibility that a complete lipoxygenase gene has been obtained. The search for a full length clone from a rabbit cDNA library is underway as its availibility would facilitate future research.

The human genomic Southern analysis was carried out primarily to verify the authenticity of the cloned human DNA in λ, as there was concern about the possible recombinational events between 2 different DNA molecules that infect the same *rec* A^+ bacterial cell,

resulting in extensive rearrangement of the cloned DNA. Repetitive sequences, like the *Alu* elements present in the human genome (Jelinek *et al.*, 1980), increase the probability of homologous recombination. The comparison of the MR7-*Xba* I fragment hybridization to the cloned human DNA and the genomic DNA would, in all likelihood, identify any alterations. From the evidence depicted in Figures 13 and 14, the 3 clones seem to represent unmodified human DNA sequences. All the restriction fragments hybridizing to the cDNA probe are accounted for in the genomic DNA Southern transfer (Fig. 14 and Table 2).

Analysis of the lipoxygenase transcript

The lipoxygenase mRNA is detected in relatively high amounts in maturing reticulocytes, but erythrocytes are almost completely devoid of the message. The presence of lipoxygenase transcript from human and rabbit reticulocytes and the T-lymphoma cell-line Jurkat RNA pools has been analyzed in this study.

Rabbit reticulocyte lipoxygenase cDNA probe selects a 2.5 kb mRNA from both human and rabbit reticulocyte poly(A)$^+$ fractions (Figs. 17 and 18). The chain length (in nucleotide residues) of the lipoxygenase message has been calculated to be between 2470 and 2647 as deduced from electron-microscopy and sucrose gradient centrifugation techniques, respectively. By polyacrylamide gel electrophoresis, the size of this transcript has been estimated at 3400 bases (Thiele *et al.*, 1982). When a human genomic probe was employed to detect the lipoxygenase transcript from the same RNA sources, the 2.5 kb message

that hybridizes to the probe was identical in size to the lipoxygenase mRNA. It is therefore conceivable that the cloned human DNAs represent sequences that are transcribed, *in vivo*, into a species of RNA corresponding in size to the known lipoxygenase transcript. However, the hybridization pattern of the cell-line RNA varies considerably from probe to probe. In contrast to the absence of a transcript homologous to the MR7-*Xba* I sequences (Fig. 17 lane 2), the λ3-B4 3.35 kb *Eco* RI fragment apparently binds the total RNA in a non-specific manner (Fig. 18 lane 2). The ambiguity with respect to the cell-line RNA:human DNA probe hybridization is explained by proposing that the 3.35 kb human DNA fragment contains sequences from a repetitive element. Several families of repeating elements exist in the human genome, including the Long Interspersed Sequence One (L1) family of genes which are reported to consist of potential protein-coding sequences (Burton *et al.*, 1986). This element is found in all mammalian genomes. The presence of *Alu* sequences is proposed in the light of the results from the human genomic Southern transfer probed with the same fragment (Fig. 19), showing that the repeat element is unique to the human DNA. It has been shown that human *Alu* sequences exist as a subset of heterogeneous nuclear RNA (hnRNA) molecules (Jelinek *et al.*, 1980) and this can explain the result obtained in Figure 18, lane 2. However, no conclusive evidence was obtained to confirm specifically the presence of *Alu* sequences. Subsequently, a cloned human DNA fragment was sought without the repetitive sequence (300 bp *Bgl* II fragment from λ3-B4) and used to probe both Northern blots. The results are depicted in Figure 20 (A and B). The absence of the lipoxygenase transcript in the human

T-lymphoma cell-line is not surprising; in a study by Goldyne *et al.* (1984), detection of 5-lipoxygenase products was attributed to contamination from a monocyte culture. The observations in this work seem to suggest that there is an absence of lipoxygenase synthesis in the human T-lymphocyte cell-line Jurkat.

The appearance of an arachidonic acid 15-lipoxygenase pathway is observed in a human promyeloma, HL-60, following the differentiation of the cell-line (Lundberg *et al.*, 1985). This cell-culture originates from peripheral leukocytes of an adult female with acute promyelocytic leukemia (Collins *et al.*, 1977). Since promyelocytes are progenitors of all non-lymphoid blood cells including platelets (Harrison, 1982), it is suggested that a single cell system might be responsible for the generation of the lipoxygenase products (Lundberg *et al.*, 1985). It should be noted that since cell-lines are transformed and function abnormally, the T-lymphoma under study may not represent the regular physiological processes of lymphocytes.

Diversity of the lipoxygenase action

It is reported that the activity of 5-, 12- and 15-lipoxygenase of blood cells resides in different proteins (Rouzer *et al.*, 1985) regardless of the fact that they all mediate oxygen insertion at double bonds of fatty acids. The specificity of each enzyme could relate to the position of the double bond to be peroxidized, the substrate, or both. Bryant *et al.* (1982) demonstrated that purified rabbit reticulocyte lipoxygenase exhibits dual positional specificity (C-12 and C-15) for a selected group of polyunsaturated fatty acids. The heterogeneous COOH terminus of the enzyme as reported by Rapoport *et*

al. (1979) seemed to be the result of endogenous carboxypeptidase action leading to the formation of isozymes responsible for the change in positional activity. Later, Kuhn *et al.* (1983) established that the two activities are found in a single enzyme and that the separation of the two was not possible by isoelectric focusing. The occurrence of a lipoxygenase specific for two positions of the fatty acid substrate is further supported by the report that lipoxygenase products from potato tubers include both 5 and 8-hydroperoxy acids (Shimizu *et al.*, 1984).

Purified rabbit reticulocyte lipoxygenase exhibits leukotriene A4 synthetase activity (Bryant *et al.*, 1985), a property in common with the human leukocyte 5-lipoxygenase (Rouzer *et al.*, 1986) but differing in positional activity. Hence, enzymes from two different sources can utilize the same substrate but there are instances in which a single cell type exhibits two distinct lipoxygenase activities. In human polymorphonuclear leukocytes, two soluble lipoxygenase activities from the cytosol have been characterized. Arachidonyl 5-lipoxygenase and linoleic acid lipoxygenase represent separate enzyme entities - Ca^{+2} is required for maximal activity of both, but w-6 linoleic lipoxygenase remains partially active in the presence of EGTA. The high degree of specificity for the w-6 position of linoleic acid is similar to that of soya bean and rabbit reticulocyte lipoxygenase as shown by the presence of 13-OOH and 13-OH linoleic acid products. There is also pH optima variation (Soberman *et al.*, 1985). To make a conclusion as to the relationship of different lipoxygenases is a difficult task at this stage; however, it is probable that all lipoxygenase enzymes have in common a functional domain with variability in other regions of the

protein molecule. It is yet to be determined whether this variability corresponds to heterogeneity at the level of the genome or if it is due to post-translational modifications (glycosylation, phosphorylation, proteolytic action, etc.).

Studies at the DNA level

The purpose of this study was to clone and characterize the human lipoxygenase gene from a genomic library, considering that further research in this direction would assist in the elucidation of the complex relationships among various lipoxygenases. Immediate efforts, however, should be concentrated on a more comprehensive identification of the lipoxygenase clones at hand by exon/intron mapping (using S1 nuclease) and eventually sequencing the gene. One of the pre-requisites is of course the availibility of a full-length cDNA, which would concurrently open avenues for expression studies. Alternatively, regulation of a well-defined genomic lipoxygenase clone can be examined *in vitro* in certain mammalian cell-lines (for example, HeLa cells).

An aspect of this work that deserves attention and could prove to be very interesting is the result obtained from the human and rabbit genomic DNA Southern blots. The transfer was carried out initially to identify any DNA rearrangements in the isolated human lipoxygenase clones. Apart from hybridizing bands (homologous to the rabbit lipoxygense cDNA) that were identical to the cloned human DNA, certain novel bands were detected (Table 2). The initial assumption was that perhaps the restriction endonuclease digestions of DNA samples

from both sources were partial. After further consideration, this possibility was ruled out for several reasons: Electrophoretic analyses indicate complete digestion of the DNA samples. Moreover, the sizes of the bands with weak signals (Fig. 14) do not correspond to the sum of smaller bands with extended homology to the probe. Finally, the appearance of several weakly hybridizing bands has been shown in similar genomic Southern transfer of rabbit DNA (Dan Gietz, pers. comm.). Even though there is an obvious homology between the human and rabbit lipoxygenase genes, the interspecies diversity can be concluded from the comparison of the genomic DNA restriction pattern. Evidently, there are regions in the human and rabbit genome that share homologous sequences with the lipoxygenase cDNA to a lesser degree than others. This observation is also noted in Southern transfer of cloned human lipoxygenase, probed with the same fragment (Fig. 13, 1 and 2).

The existence of pseudogene(s) could be suggested as an alternative explanation to account for the results obtained. These inactive counterparts of functional genes have, at some point, lost the ability to synthesize protein and are therefore subject to accelerated accumulation of mutations. A pseudogene structure was initially described in the tandemly-repeated genes of 5S RNA from *Xenopus laevis* ; the homologous sequence resembles closely the gene itself but is inactive (Jacq *et al.*, 1977). The human β and α globin gene clusters are known to have 1 and 2 pseudogenes, respectively (Little, 1982). It is likely that a duplication of the ancestral lipoxygenase gene could have resulted in the formation of an inactive gene.

In retrospect, it seems only appropriate to propose that perhaps the diversity of the lipoxygenase protein is the reflection of the heterogeneity that is encountered at the DNA level. Preliminary as the data available may be, the possibility of a multigene family for lipoxygenase is, nevertheless, exciting and encourages further investigation of the area. As a first approach, *in situ* chromosome mapping would identify region(s) of the human (or rabbit) genome that correspond to the lipoxygenase gene(s). This cytological mapping, using the human probe now available, could determine whether or not there are a number of discrete lipoxygenase genes, and if so, whether they are randomly dispersed throughout the genome or exist as a clustered gene family as is the case with rRNA genes of almost all eukaryotes. One can further speculate that a "variant" gene family (such as the actin multigene family) can define the organization of lipoxygenases in the genome.

The possibilities await exploration.

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